

## CALCIUM SULFATE DEPOSITS ASSOCIATED WITH NEEDLE SUBSTOMATAL CAVITIES OF CONTAINER-GROWN LONGLEAF PINE (*PINUS PALUSTRIS*) SEEDLINGS

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Extracellular calcium sulfate ( $\text{CaSO}_4$ ) formations associated with substomatal cavities of longleaf pine (*Pinus palustris* Mill.) are described. Longleaf pine seedlings were grown with two levels of soil nitrogen (N) (40 or 400 kg N ha<sup>-1</sup> yr<sup>-1</sup>) and water stress (–0.5 or –1.5 MPa xylem pressure potential) in open-top field chambers under two levels of atmospheric CO<sub>2</sub> (365 or 720  $\mu\text{mol mol}^{-1}$ ). Needles were subjected to scanning electron microscopy after 12 mo exposure to experimental conditions. Crystalline to fibrillar formations, appressed to surfaces of guard cells facing the interior of the needle, were observed in all treatments. In some cases, both crystalline and fibrillar formations were observed to occur within the same needle cross section. Formations were characterized as calcium sulfate using energy-dispersive spectrometry. Crystal-like  $\text{CaSO}_4$  appeared to originate from guard cells in the vicinity of the stomatal aperture. Formations may arise from evaporation of plant water at the interface between stomatal antechambers and substomatal cavities, leaving Ca and SO<sub>4</sub> behind to precipitate. Many questions remain regarding their ecological and physiological significance as well as their occurrence and prevalence in both time and space.

**Keywords:** *Pinus palustris*, longleaf pine, calcium sulfate crystals, calcium oxalate crystals.

### Introduction

Calcium is required for the growth of all plants. However, within cytoplasm, Ca above micromolar concentrations is toxic; Ca may interfere with calcium-dependent signaling, phosphate-based energy metabolism, and microskelatal characteristics (Webb 1999). Plant cells avoid such detrimental effects by maintaining cytoplasmic Ca at very low levels (ca. 10<sup>-7</sup> M) (Webb 1999). Calcium is an immobile element, and amounts in excess of that required for optimal metabolic function are precipitated in the form of insoluble calcium salts such as oxalate, carbonate, sulfate, phosphate, silicate, citrate, tartrate, and malate (Franceschi and Horner 1980).

Since Leeuwenhoek described raphides within cells of *Arum* plants in a letter in 1675 (Arnott and Pautard 1970), crystals have often been observed and their significance often speculated upon. Although crystalline Ca salts are taxonomically ubiquitous and have been observed within most plant tissue types, their mechanisms of formation and their functions are still not well understood. However, several functions have been attributed to crystals within plants, including defense against foraging animals (Doaigey 1991), precipitation of potentially toxic waste products (Franceschi 1984), and control of cellular ionic and osmotic balance (Franceschi and Horner 1980). Crystals have also been postulated to provide structural support (Okoli and Mceuen 1986; Pritchard et al. 1997b).

Crystals of calcium oxalate and calcium carbonate are un-

doubtedly the most prevalent and most extensively studied of all Ca salts found in plants. These salts occur in angiosperms, gymnosperms, and pteridophytes, usually forming within cell walls or vacuoles. In contrast, calcium sulfate ( $\text{CaSO}_4$ ) deposits are rare in plants, having been described in only a few cases (Arnott and Pautard 1970; Franceschi and Horner 1980; Storey and Thomson 1994). For example,  $\text{CaSO}_4$  crystals have been reported in pith (Arnott and Pautard 1970) and mesophyll vacuoles (Storey and Thomson 1994) of *Tamarix* (evergreen athel). Calcium sulfate crystals have also been reported in *Capparis* (caper; Arnott and Pautard 1970). However, a later study reported that the crystals found within ray cells of secondary xylem in *Capparis* are of a composition previously unknown, consisting of 20%–25% Ca, 10%–15% K, and 15%–20% S (Miller 1978). Calcium sulfate crystals on external needle surfaces of conifers have been reported as a symptom of acid rain treatments in several studies (Huttunen et al. 1991; Fink 1991b).

To our knowledge,  $\text{CaSO}_4$  crystals have not been reported within needles of any pine species. In a thorough microscopical study of patterns of calcium oxalate production in needles of various conifer species (Fink 1991a), no mention was made concerning the occurrence of  $\text{CaSO}_4$  crystals for any of the 26 species examined. Furthermore, it was concluded by Fink (1991a, p. 306) that “During these studies it became evident, however, that there still exists a great lack of knowledge about the ‘normal’ structure and physiology of conifer needles. ... That seems to be especially true for the distribution and functioning of mineral elements.” Therefore, the purpose of this report is to describe the extracellular crystalline  $\text{CaSO}_4$  formations observed within substomatal cavities of longleaf pine (*Pinus palustris* Mill.) needles. The  $\text{CaSO}_4$  deposits described here were discovered during a comprehensive investigation

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into the effects of elevated atmospheric CO<sub>2</sub> concentrations on growth, physiology, ultrastructure, anatomy, and morphology of longleaf pine seedlings.

## Material and Methods

### *Plant Exposure System*

Longleaf pine seedlings (mean root collar diameter = 13 mm, SD = 2) from a wild seed source were exposed to elevated CO<sub>2</sub> (ca. 720  $\mu\text{mol mol}^{-1}$ ) and ambient CO<sub>2</sub> (ca. 365  $\mu\text{mol mol}^{-1}$ ) conditions in open-top chambers (Rogers et al. 1983) beginning March 30, 1993, and seedlings were maintained until the final harvest on November 28, 1994. The chambers, CO<sub>2</sub> supply, and CO<sub>2</sub> monitoring/dispersing systems have been described by Mitchell et al. (1995).

Seedlings were planted in a coarse sandy medium (pH 5.1) in 45-L containers. Two levels of soil nitrogen and water were employed. Nitrogen treatments (applied as sulfur-coated urea, 38 : 0 : 0) consisted of 4 g m<sup>-2</sup> yr<sup>-1</sup> for the low treatment and 40 g m<sup>-2</sup> yr<sup>-1</sup> for the high treatment and were administered according to Mitchell et al. (1995). Other nutrients were maintained at nonlimiting levels by application of sulfur-coated potassium (0.04 mg K g<sup>-1</sup> soil yr<sup>-1</sup>) and MicroMax Plus (P = 0.14, Ca = 0.57, Mg = 0.28, and S = 0.05 mg g<sup>-1</sup> soil yr<sup>-1</sup>, plus a complete complement of micronutrients). In April 1993, a single application of iron chelate (0.007 mg Fe g<sup>-1</sup> soil) was made.

After seedling initiation (19 wk after start of the study), Teflon rain-exclusion caps were fitted to chambers in order to implement different soil-water regimes. Water-stressed plants were allowed to dry to -1.5 MPa before watering, and well-watered plants were maintained between 0 and -0.6 MPa predawn xylem pressure potential. Xylem pressure potentials were determined periodically throughout the study with a pressure bomb (Scholander et al. 1965). Water status determined from the pressure bomb was converted into gravimetric standards so that appropriate water regimes could be maintained, using a pneumatic weighing device of our own design.

### *Scanning Electron Microscopy*

Needle tissues were collected in March 1994 after 12 mo exposure to the various treatments; seedlings were 18 mo old at the time of sampling. Needles were selected from fully expanded three-needle fascicles from seedlings representing all possible combinations of nitrogen and water treatments from two chambers supplied with ambient CO<sub>2</sub> and two amended with twice-ambient levels. Fully expanded needles were selected from the bottom one-third of seedlings such that needles had developed to maturity entirely under experimental conditions (the cohort was tagged immediately after initiation the previous spring). Needle segments (2–3 mm) were excised from the center portion of needles and fixed in formalin–acetic acid–alcohol. After 24 h, the segments were dehydrated in an ethanol dehydration series, critical-point dried, and sputter-coated with gold–palladium (SC-7, Ted Pella, Redding, Calif.). Multiple needle segments (4–7 per treatment  $\times$  8 treatments = 32–56) were viewed from two replications of all treatments. Segments were photographed with a scanning electron microscope (SEM; DSM

940 Zeiss, Oberkochen), and elemental composition of crystalline formations was accomplished with attached energy-dispersive spectrometry (EDS; Tracor Northern, Middleton, Wis.). Elemental quantification was done on a Jeol SEM coupled to an Oxford EDS system operating at 20 kV. Standards for O, S, and Ca were quartz, FeS<sub>2</sub>, and Wollas, respectively.

### *Sulfur Analysis*

Concurrent with collection of plant tissue for SEM, leaf tissue was collected from plants representing all combinations of CO<sub>2</sub>, N, and water treatments, dried to a constant weight at 55°C, and ground to pass through a 2-mm screen. Soil samples from all pots were also collected, dried, and ground. Total S content of leaves and soil was determined with a LECO CHN-600 analyzer (LECO, Augusta, Ga.) and is expressed as percent of total sample weight.

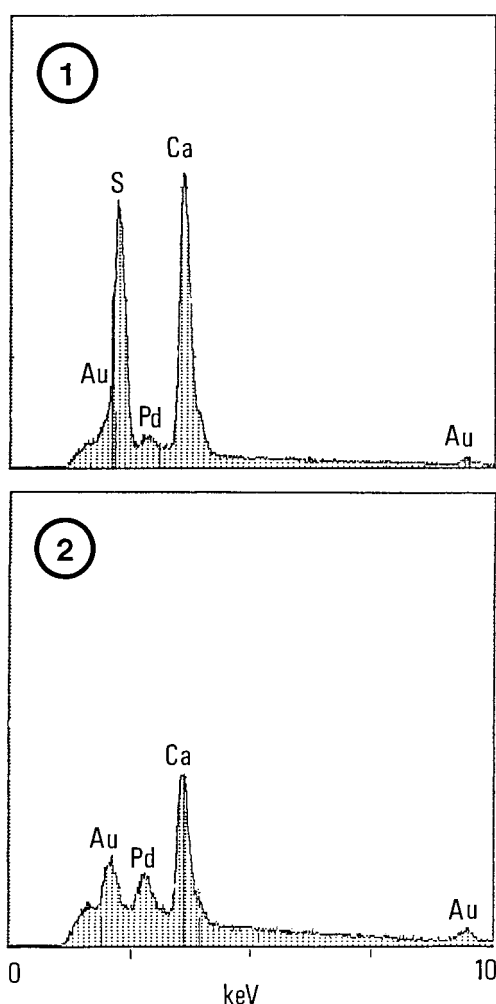
Leaf tissue was harvested and soil samples were collected from a naturally occurring xeric and hydric site in a longleaf pine ecosystem typical of the Tuskegee National Forest in Tuskegee, Alabama. We chose plants that were similar in age and size to those grown under experimental conditions. Leaf tissue from seedlings growing in both mesic and hydric sites was collected in order to compare the S content of soil and leaves from nature with those of the container-grown experimental seedlings. Needle S contents of plants grown in pots and plants grown in nature were compared using ANOVA (SAS Institute 1996). Pairwise comparisons were made using Fisher's protected least significant difference (PLSD) test (SAS Institute 1996). Differences were considered significant at the  $\alpha \leq 0.05$  level.

## Results

CaSO<sub>4</sub> deposits were observed in needles from seedlings representing all possible permutations of elevated- and ambient-CO<sub>2</sub>, high- and low-N-availability, and adequately watered and water-stressed treatments. Thus, it appears that resource availability does not affect the incidence or prevalence of these deposits. Furthermore, formations did not appear to be distributed differently on flat compared to curved needle surfaces. However, it is important to note that the numbers of CaSO<sub>4</sub> deposits were sporadic in their occurrence; many needle cross sections did not contain these deposits, and of those that did, no cross section had more than three visible deposits present.

Energy-dispersive spectrometry indicated a composition of Ca, S, and O (fig. 1.1). Although O is not shown on these spectra derived from the Tracor Northern EDS system, the O peak was obvious on the spectra from the more sensitive Jeol EDS system (data not shown). Quantitative analysis revealed atomic percentage values of 54%, 21%, and 25% for O, S, and Ca, respectively. These values indicate a stoichiometry that is consistent with CaSO<sub>4</sub>. Spectra indicated that the background signal (hypodermis) adjacent to the CaSO<sub>4</sub> formations contained Ca but no detectable S (fig. 1.2), as was also the case with mesophyll cells (data not shown). The stalks of the formations did not differ in composition from the main deposits.

Deposits were situated extracellularly, appressed against the surfaces of the guard cells, protruding into substomatal cavi-



**Fig. 1** Spectra from energy-dispersive spectrometry of longleaf pine needles. Gold (Au) and palladium (Pd) were used to sputter-coat the samples for viewing with the SEM. Fig. 1.1, Typical spectrum from a  $\text{CaSO}_4$  formation (pictured in figs. 2.4, 2.5), showing large calcium (Ca) and sulfur (S) peaks. Fig. 1.2, Typical spectrum from area adjacent to the  $\text{CaSO}_4$  formations, showing a Ca peak and no S peak.

ties. They often appeared to hang from stalks that were holding them in place between guard cells (figs. 2.3, 2.6, 2.7; figs. 3.9, 3.10), although in some cases the deposits were dislodged, presumably during specimen preparation (figs. 2.4, 2.6). Calcium sulfate deposits were observed neither in stomatal antechambers nor intracellularly.

The consistency of the structures varied from very fine, homogeneous, and rodlike (figs. 2.4, 2.5) to rodlike, with larger angular crystals embedded within (figs. 2.7, 2.8). Deposits of intermediate appearance were also noted (fig. 3.10). The fibrillar strands appear to radiate from a central location within the formations at the site of contact between the two guard cells (figs. 2.4, 2.6). These  $\text{CaSO}_4$  deposits often appeared cracked, but we are unsure if this occurred as a result of fixation or from heating under the electron beam in the SEM (figs. 2.5, 2.7). Deposits varied in size from ca. 7 to 56  $\mu\text{m}$  in diameter.

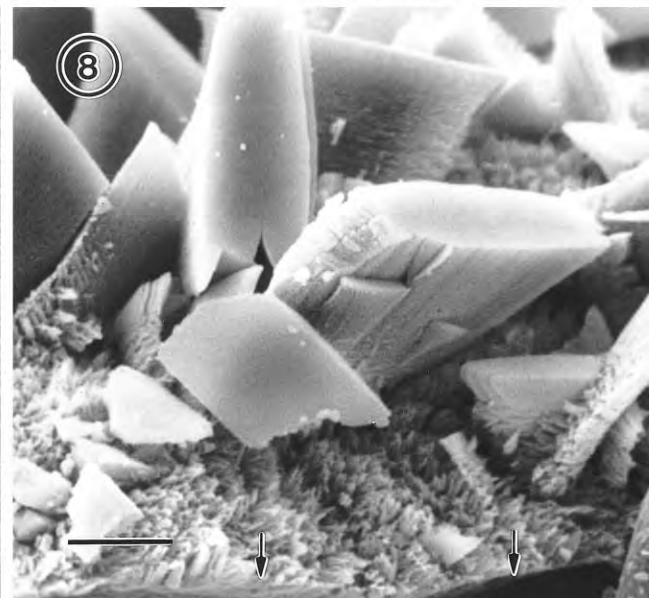
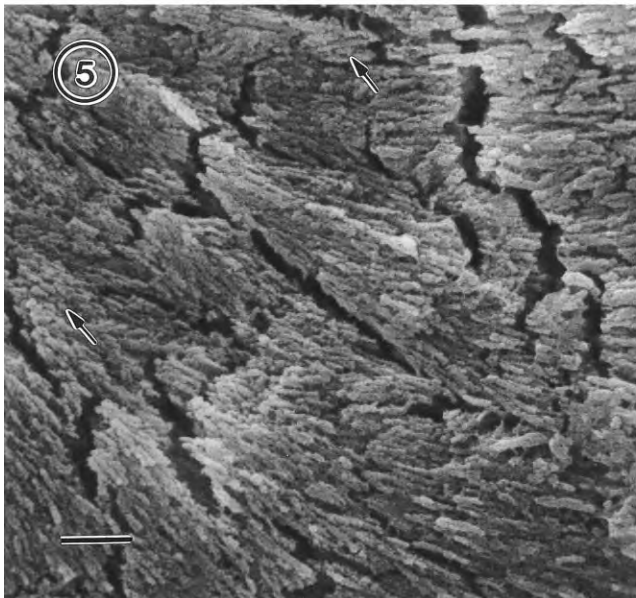
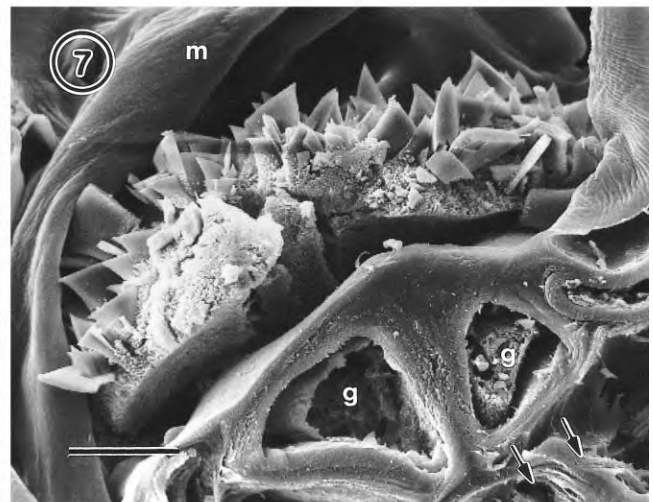
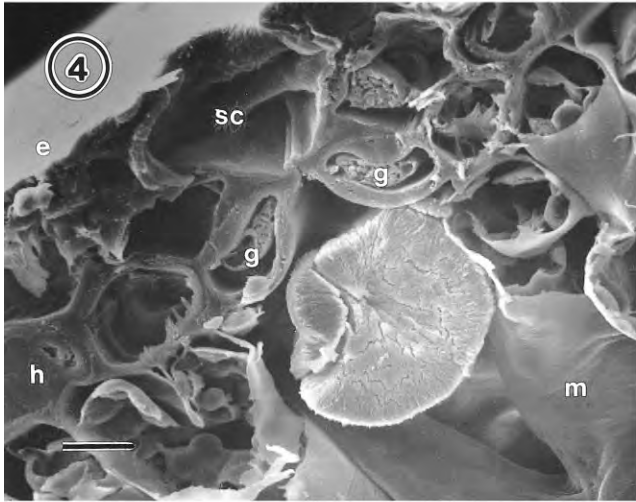
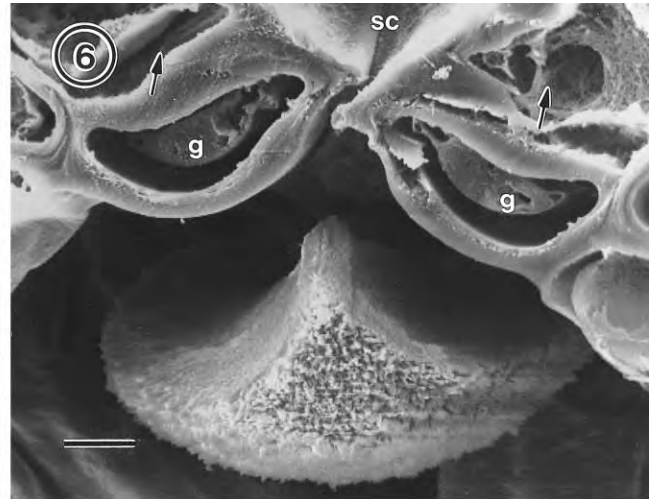
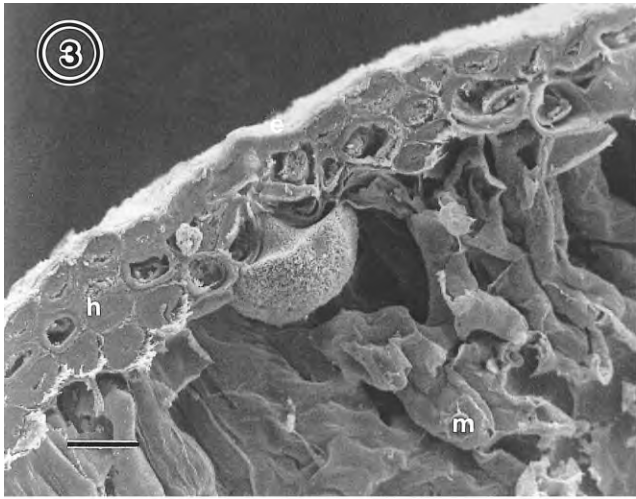
Analysis of leaf S showed that S content from plants grown under the experimental treatments was generally higher than the S content of the leaves from plants growing in nature. However, there was no statistical difference between the S content of leaves from the hydric natural site and those from several of the experimental treatments (fig. 4A). For example, there were no statistical differences between the S content of needles from the hydric location and those from the  $\text{eCO}_2\text{-HN-WW}$ , the  $\text{aCO}_2\text{-LN-WW}$ , or the  $\text{eCO}_2\text{-LN-WS}$  treatment combinations (see fig. 4 legend). Therefore, although it was generally lower, the S content of leaves exhibiting the  $\text{CaSO}_4$  formations from the experiment are not out of the range found in nature. Sulfur content in soil sampled from the container-grown seedlings did not differ from that of the soil collected at the two natural sites ( $P = 0.6053$ ; fig. 4B).

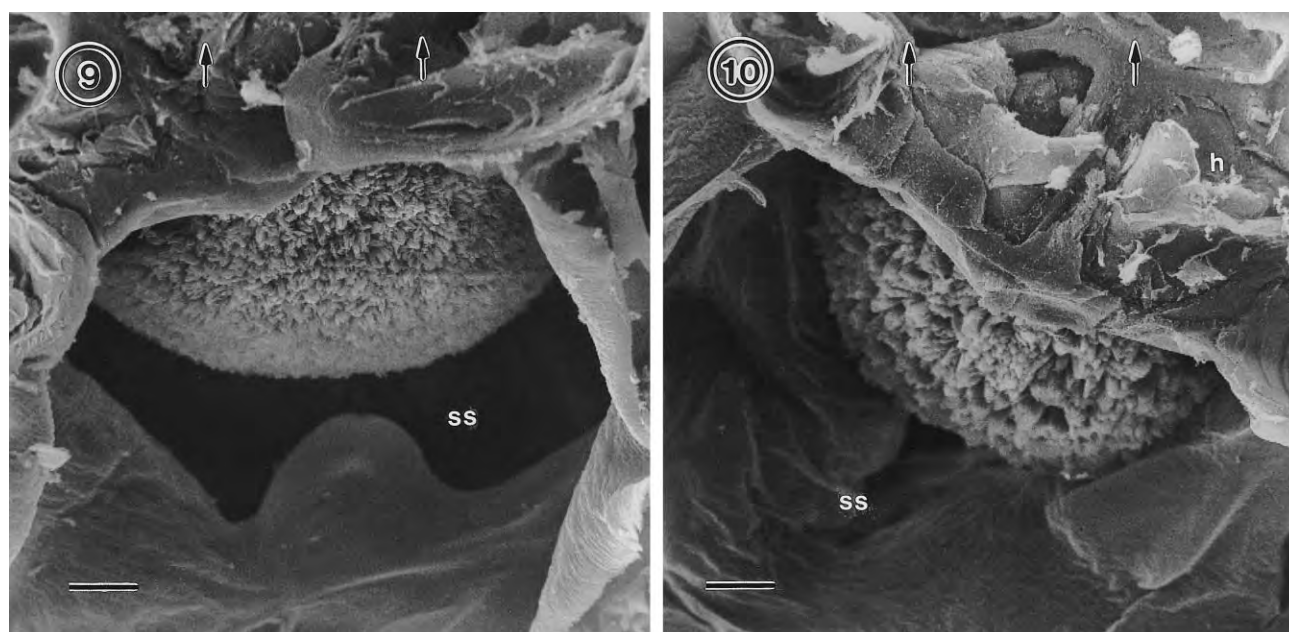
## Discussion

The  $\text{CaSO}_4$  formations described herein were observed during a comprehensive multidisciplinary project intended to characterize the effects of elevated atmospheric  $\text{CO}_2$  on longleaf pine grown under conditions of varied N and water availability. Data from these same seedlings have been published elsewhere (growth and biomass, Prior et al. 1997b; photosynthesis, Runion et al. 1999b; surface wax deposition, Prior et al. 1997a; starch grain, calcium oxalate, and tannin contents, Pritchard et al. 1997b; leaf ultrastructure, Pritchard et al. 1997a; leaf anatomy, Pritchard et al. 1998; respiration, Mitchell et al. 1995; tissue chemistry, Runion et al. 1999a and Entry et al. 1998; and mycorrhizae, Runion et al. 1997).

In angiosperms, crystals of calcium oxalate or calcium carbonate almost always occur intracellularly, often within specialized cells (i.e., crystal idioblasts, lithocysts). In pine species, calcium oxalate crystals may be located both intracellularly or extracellularly (Franceschi and Horner 1980; Fink 1991a). Intracellular calcium oxalate crystals within ray cells of needle phloem have been previously reported in longleaf pine (*Pinus palustris* Mill.) (Pritchard et al. 1997b). However, extracellular calcium oxalate crystals are either not present or are present only rarely (S. Pritchard, personal observation). Furthermore, there are no previous reports of naturally occurring  $\text{CaSO}_4$  crystals within tissues of any pine species.

In another study in which  $\text{CaSO}_4$  crystals were found on the plant exterior, Turunen et al. (1995) reported the presence of  $\text{CaSO}_4$  crystallites on needle surfaces of *Pinus sylvestris* L. (Scotts pine) and *Picea abies* (L.) Karst. (Norway spruce) in response to acid rain treatments. They found that the presence and numbers of crystals on the needle surface increased with increasing duration of the acid rain treatment. In order to simulate the effects of acid deposition, these investigators added  $\text{H}_2\text{SO}_4$  and  $\text{HNO}_3$  to tap water containing 23.8 mg Ca  $\text{L}^{-1}$ , 24.9 mg  $\text{SO}_4$   $\text{L}^{-1}$ , and 0.27 mg  $\text{NO}_3$   $\text{L}^{-1}$  until the pH dropped to ca. 3.0. They concluded that the formation of crystals was facilitated by leaching of needle Ca as a result of increased cuticular permeability. Furthermore, although they did observe that in a few cases the crystals appeared to be extruded from stomatal antechambers, they concluded that the S in the crystals was derived directly from the acid rain treatments. Adams et al. (1990) also found  $\text{CaSO}_4$  crystallites on cabbage leaf surfaces as a direct consequence of an acid rain





**Fig. 3** Scanning electron micrographs of cross sections of longleaf pine needles showing undisturbed  $\text{CaSO}_4$  formations hanging from the juncture of guard cells. Arrows point in the direction of the needle surface. Bars = 5  $\mu\text{m}$ .

treatment. In this study, longleaf pine was irrigated with deionized water in order to avoid mineral interference with proper plant nutrition. Furthermore, few extra S-containing compounds were added to the ambient air as a result of the  $\text{CO}_2$  treatments. The pure liquid  $\text{CO}_2$  (>99%) used in this study contained less than 0.1% total S compounds, and this small addition was further diluted to achieve the target  $\text{CO}_2$  concentrations of 720  $\mu\text{mol mol}^{-1}$ . These formations were also observed in both ambient and elevated  $\text{CO}_2$  treatments, thus indicating that there is no effect of the  $\text{CO}_2$  addition on  $\text{CaSO}_4$  deposition. Use of demineralized water in conjunction with the location of  $\text{CaSO}_4$  formations in the interior of the needle indicates an endogenous origin for the  $\text{CaSO}_4$  structures observed here.

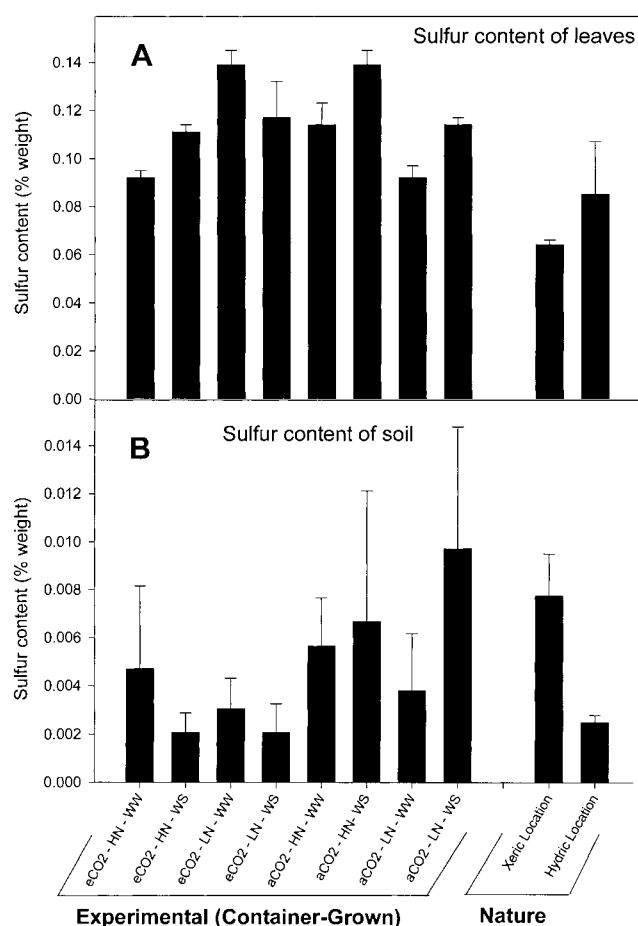
It is possible that the plants used in this experiment were supplied with excess S; N and K were both applied in the S-coated form in order to allow the nutrients to be released slowly over time. However, values derived from analysis of S in leaves from seedlings grown in the experiment were not out of the range of the S content of leaves found in nature from seedlings similar in age and size. Furthermore, the S content of the soil of the container-grown experimental plants did not differ from the S content of the soil collected from nature. This

leads us to believe that the amount of S available to these plants was not unrealistic. Future experiments are being planned in which longleaf pine will be grown in soils exhibiting a gradient in S content in order to elucidate whether or not this phenomenon is associated with high-S soils only.

One could speculate that the  $\text{CaSO}_4$  formations would not be expected to occur in plants supplied with the lower N level for three reasons: (1) the plants in the low-N treatment received one-tenth the amount of S (as a result of the N application) received by the high-N plants; (2) N-limited plants exhibited lower rates of photosynthesis (Runion et al. 1999b) and lower growth rates (Prior et al. 1997b), which may have adversely affected root protein contents (i.e., carrier proteins) and energy available for uptake; and (3) reduced passage of S into the plant through the transpiration stream would be expected in water-stressed plants growing in low-N soil because plants were smaller (Prior et al. 1997b) and transpiration was significantly reduced (Runion et al. 1999b). Crystals, however, were found in all treatments. This indicates that the presence of these deposits was not simply an artifact of excess soil S availability.

Assuming that these  $\text{CaSO}_4$  deposits result from normal plant function, questions immediately arise concerning their

**Fig. 2** Scanning electron micrographs of cross sections of longleaf pine needles. Note that the shapes of the main crystals and stalks follow the outline of the substomatal chamber and the juncture of the guard cells. This, along with the orientation of the rodlike material, may provide further evidence for formation of these structures during transpiration. Figs. 2.3, 2.6,  $\text{CaSO}_4$  formations with stalks connecting them to the juncture of guard cells at the stomatal aperture. Fig. 2.4, Micrograph showing cross section of formation that has apparently been dislodged during specimen preparation. Fig. 2.5, High magnification of  $\text{CaSO}_4$  formation from fig. 4, showing small rodlike material with fissures. Figs. 2.7, 2.8, Low- and high-magnification micrographs showing  $\text{CaSO}_4$  formation, with large angular crystals embedded within a matrix of smaller fibrillar material. Arrows point in direction of the needle surface. Bars = 20  $\mu\text{m}$  in fig. 2.3; 10  $\mu\text{m}$  in figs. 2.4, 2.7; 1  $\mu\text{m}$  in fig. 2.5; 5  $\mu\text{m}$  in fig. 2.6; and 2  $\mu\text{m}$  in fig. 2.8. Abbreviations: *e* = epidermis; *h* = hypodermis; *m* = mesophyll; *g* = guard cell; *sc* = stomatal crypt; *ss* = substomatal space.



**Fig. 4** Graphs showing the sulfur content of leaves (A) and soil (B) from experimentally grown plants and from xeric and hydric sites in nature. Bars indicate standard error of the mean. eCO<sub>2</sub> = 720  $\mu\text{mol mol}^{-1}$ ; aCO<sub>2</sub> = 365  $\mu\text{mol mol}^{-1}$ ; HN = high-N availability; LN = low N availability; WW = well watered; WS = water stressed.

ontogeny and significance. It is interesting, and perhaps functionally significant, that the  $\text{CaSO}_4$  formations were situated at the end of the transpirational stream, where plant water evaporates and is transpired through stomata. It is thought that plant Ca and  $\text{SO}_4$  are carried along the transpirational stream apoplastically and are exchanged with cells at various points along the way. In many plants, minerals in excess of metabolic requirements remain in the transpirational stream and are eventually expelled via guttation, leaching, and abscission (Arnott and Pautard 1970). Guttation is thought to be a very general phenomenon in plants, one that occurs through hydathodes, stomata, and lenticels. Guttation may lead to injury of leaf margins in some plant species as the result of salt deposits left behind by guttation fluid. However, it is thought that guttation does not occur in pine species because

of the absence of root pressure (Kozlowski and Pallardy 1997). Perhaps in longleaf pine, significant amounts of Ca and  $\text{SO}_4$  are left behind at the margins of the guard cells, where plant water changes state from liquid to gas when exposed to the less humid conditions prevailing within stomatal crypts. The evaporation of large amounts of water at this interface might leave behind significant amounts of minerals that were carried previously in the transpirational stream, thereby leading to precipitation of Ca with  $\text{SO}_4$ .

Functionally,  $\text{CaSO}_4$  formations blocking stomatal apertures might serve as a barrier to pathogen (i.e., fungi) entry. For example, Patton and Johnson (1970) observed that epistomatal wax plugs in *Pinus strobus* often prevented growth of germ tubes of the fungal pathogen *Cronartium ribicola* into stomatal pits, thus reducing infection of needles. Furthermore, they attributed increased incidence of plugged stomata in older needles with increased resistance to fungal infections. Calcium sulfate formations described here may have similar functions.

The orientation of  $\text{CaSO}_4$  formations against the guard cells, which apparently block stomatal apertures, may have significant implications for plant-water relations and photosynthesis. These formations may functionally increase resistance to gas exchange by altering the physical characteristics of the diffusive pathway. These formations might function as anti-transpirants analogous to wax deposited within stomatal crypts of many pine species (*P. sylvestris*, Hanover and Reicosky 1971; *Pinus nigra*, Reicosky and Hanover 1976; *P. strobus*, Johnson and Riding 1981; and *P. palustris*, Prior et al. 1997a). Crystalline wax within stomatal antechambers was estimated to reduce transpiration by two-thirds while reducing photosynthesis by only one-third in *Picea sitchensis* (Jeffree et al. 1971). Perhaps the  $\text{CaSO}_4$  formations observed in longleaf pine serve a similar function, thereby contributing to longleaf pine's classical xeromorphy. Such a function would be of considerable adaptive value in light of longleaf pine distribution at the more xeric end of the moisture continuum in the southeastern United States. However, we must conclude by stating that many questions remain regarding the prevalence and distribution of substomatal  $\text{CaSO}_4$  depositions within pine needles in both time and space.

### Acknowledgments

We thank Barry G. Dorman, Tammy K. Counts, Leigh Crouse, and Cecilia Mosjidis for technical assistance and G. Brett Runion, Micheal Davis, and Roland R. Dute for reviewing an earlier draft of this manuscript. This material is based on work supported through the Southeastern Regional Center of the National Institute for Global Environmental Change by the U.S. Department of Energy under Cooperative Agreement DE-FC03-90ER61010 and through the Experimental Program to Stimulate Competitive Research by the U.S. Environmental Protection Agency under contract R821826-01-1.

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